Figure S1

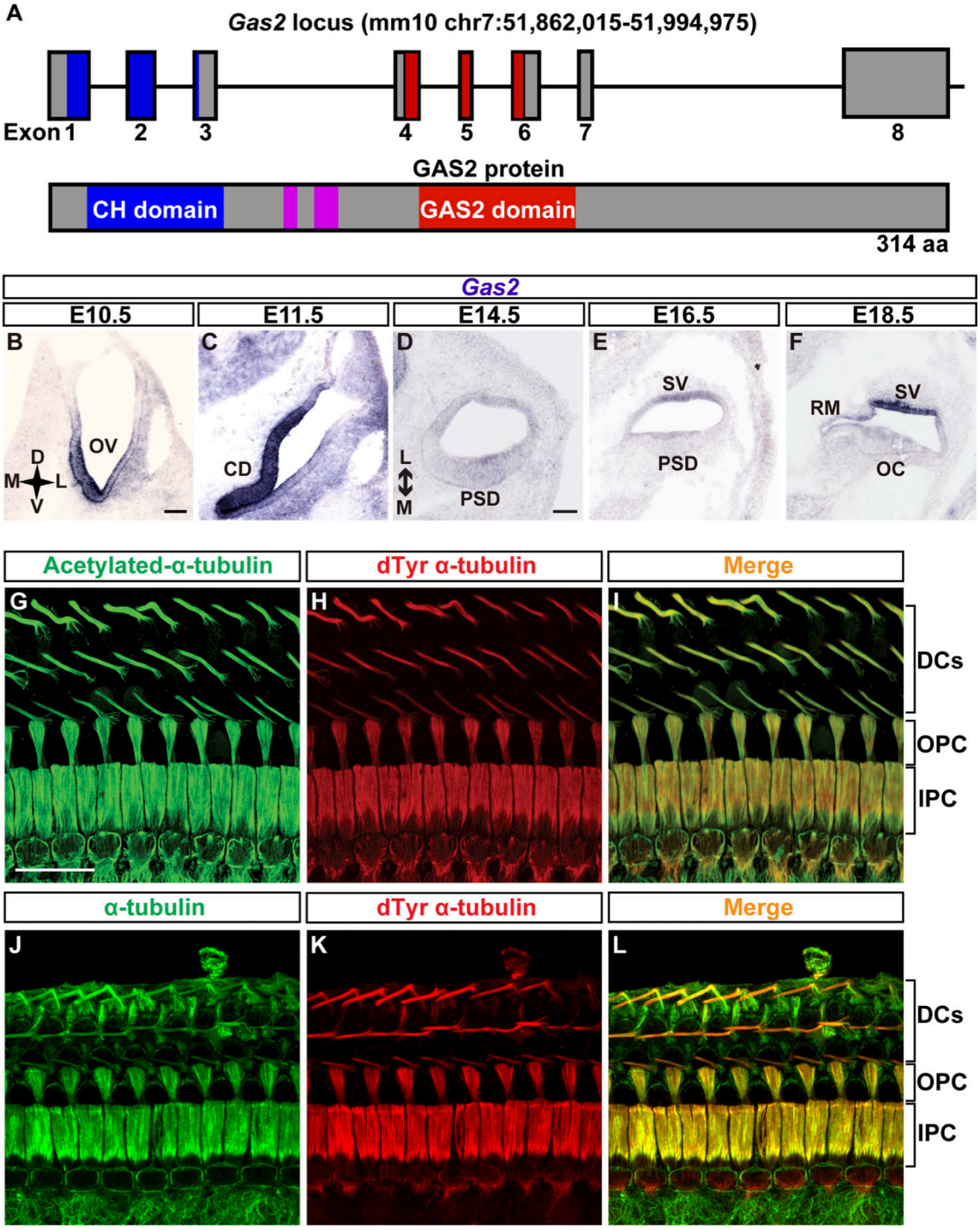
A
Gas2 locus (mm10 chr7:51,862,015-51,994,975)
Exon 1
2
3
4
5
6
7
8
GAS2 protein
CH domain
GAS2 domain
314 aa
Gas2
E10.5
E11.5
E14.5
E16.5
E18.5
B
OV
D
M
L
V
C
CD
D
L
M
PSD
E
SV
PSD
F
RM
SV
OC
Acetylated-α-tubulin
dTyr α-tubulin
Merge
G
H
I
DCs
OPC
IPC
α-tubulin
dTyr α-tubulin
Merge
J
K
L
DCs
OPC
IPC

Figure S1. *Gas2* is expressed in the developing cochlea (related to Fig.1).

(A) Schematic of the mouse *Gas2* gene and encoded protein highlighting the positions of the calponin homology (CH) and GAS2 domains. **(B-F)** Transverse sections through the inner ear showing the spatial and temporal patterns of *Gas2* expression during mouse embryonic development. Scale bar = 100μm (B, C); 50μm (D-F). **(G-L)** Whole mount immunostaining of cochlear preparations at P14 with antibodies against acetylated α-tubulin and dTyr α-tubulin (G-I), and pan α-tubulin and dTyr α-tubulin (J-L). The majority of microtubules in support cells are both acetylated and detyrosinated. Scale bar = 20μm. Abbreviations: cochlear duct (CD), dorsal (D), lateral (L), medial (M), prosensory domain (PSD), organ of Corti (OC), otic vesicle (OV), Reissner's membrane (RM), stria vascularis (SV), ventral (V).

Figure S2

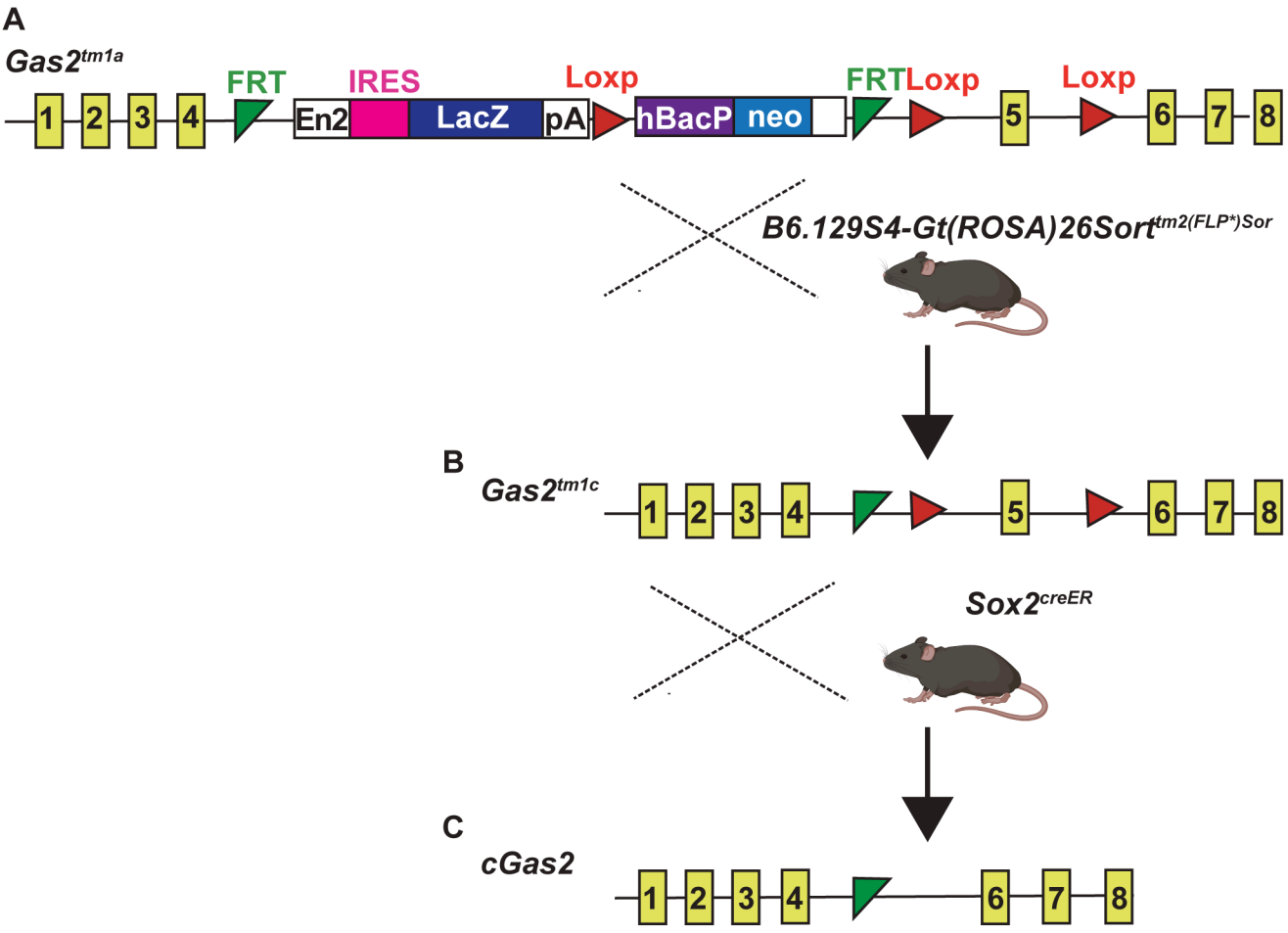

Figure S2. Breeding scheme to generate *Gas2* mutant alleles (related to Fig. 2).

(A) Schematic of the *Gas2* knockout first allele (*Gas2*^{tm1a}) generated by the European Conditional Mouse Mutagenesis Program. **(B)** The *Gas2* floxed allele (*Gas2*^{tm1c}) was generated by crossing *Gas2*$^{tm1a/+}$ mice with a FLPo deletor strain and selecting progeny with loxp sites surrounding exon 5. **(C)** Conditional *Gas2* knockout mice (*cGas2*) were generated by crossing *Gas2*$^{tm1c/tm1c}$ mice with an inducible *Sox2*^{CreER} line, which selectively deletes *Gas2* in supporting cells upon tamoxifen administration.

Figure S3

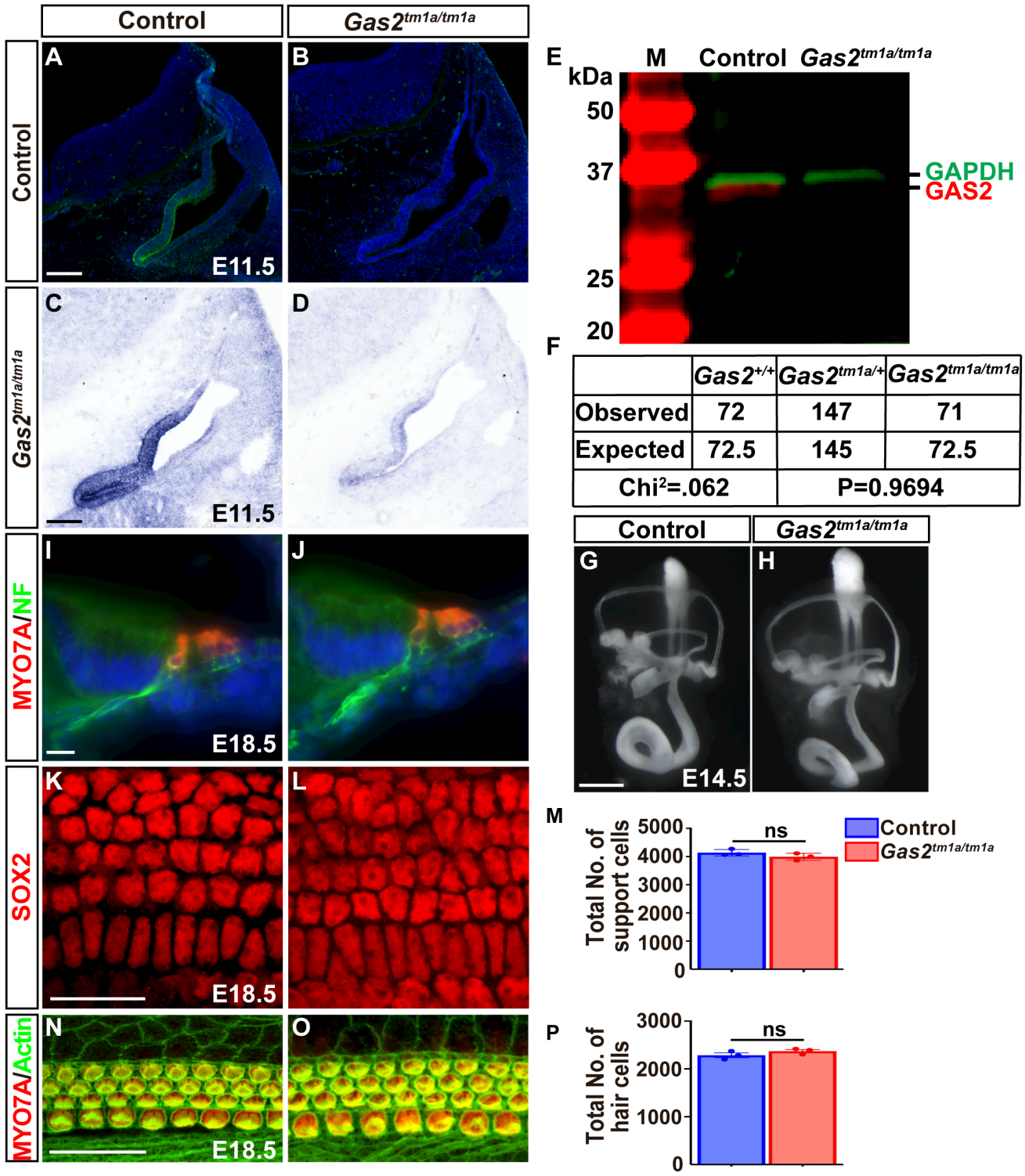
Control
Gas2tm1a/tm1a
A
B
E11.5
C
D
E11.5
I
J
E18.5
K
L
E18.5
N
O
E18.5
Control
Gas2tm1a/tm1a
MYO7A/NF
SOX2
MYO7A/Actin
E
M
Control
Gas2tm1a/tm1a
kDa
50
37
25
20
GAPDH
GAS2
F
Gas2+/+
Gas2tm1a/+
Gas2tm1a/tm1a
Observed 72 147 71
Expected 72.5 145 72.5
Chi²=.062
P=0.9694
Control
Gas2tm1a/tm1a
G
H
E14.5
M
Total No. of support cells
5000
4000
3000
2000
1000
0
ns
Control
Gas2tm1a/tm1a
P
Total No. of hair cells
3000
2000
1000
0
ns

Figure S3. Inner ear development is unaffected by loss of GAS2 (related to Fig. 2). **(A-D)** Transverse sections through the otic vesicle showing the loss of GAS2 protein (A,B) and *Gas2* mRNA (C,D) in *Gas2*$^{tm1a/tm1a}$ embryos compared to controls (n=3). **(E)** Western blot showing loss of GAS2 expression (red band) in relation to GAPDH (green band) in cochleae from *Gas2*$^{tm1a/tm1a}$ embryos compared to controls (P0) (n=3). **(F)** Table displaying the observed genotypes of progeny from *Gas2*$^{tm1a/+}$ intercrosses at the time of weaning, which fit the expected Mendelian ratios. **(G-H)** Paint fills of control and *Gas2*$^{tm1a/tm1a}$ inner ears at E14.5 revealed no difference in morphology (n=3). **(I-J)** Transverse sections through the cochlea stained with markers of hair cells (MYO7A) and spiral ganglia (NF, neurofilament) show normal patterns of innervation between control and *Gas2*$^{tm1a/tm1a}$ embryos at E18.5. **(K-P)** Whole mount immunostaining of cochlear preparations show no differences in the number of SOX2 positive supporting cells (K-M), or MYO7A, Phalloidin/F-actin positive hair cells (N-P) between control and *Gas2*$^{tm1a/tm1a}$ embryos at E18.5. **(M,P)** Quantification of the total number of hair and supporting cells is presented as mean ±SD (ns = not significant, p=0.2373 (M), p=0.2156 (P), two-sided t-test, n=3). Scale bar = 100μm (A-H), 10μm (I-J), 20μm (K-O).

Figure S4

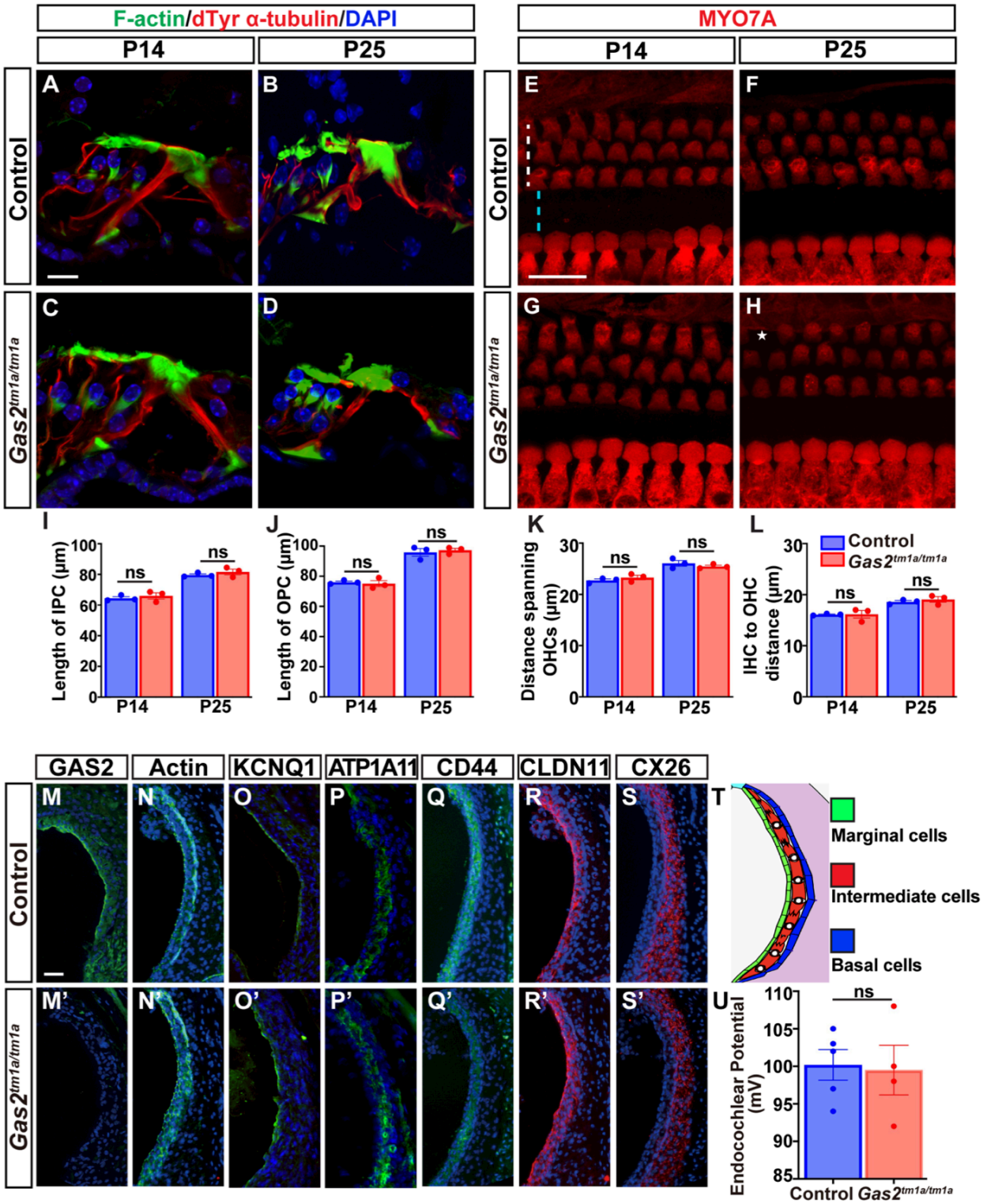
F-actin/dTyr α-tubulin/DAPI
MYO7A
P14
P25
Control
Gas2tm1a/tm1a
A
B
C
D
E
F
G
H
I
J
K
L
Length of IPC (μm)
Length of OPC (μm)
Distance spanning OHCs (μm)
IHC to OHC distance (μm)
ns
Control
Gas2tm1a/tm1a
GAS2
Actin
KCNQ1
ATP1A11
CD44
CLDN11
CX26
M
N
O
P
Q
R
S
M'
N'
O'
P'
Q'
R'
S'
T
Marginal cells
Intermediate cells
Basal cells
U
Endocochlear Potential (mV)
Control
Gas2tm1a/tm1a

Figure S4. Cellular organization in the organ of Corti and stria vascularis is maintained in postnatal *Gas2*$^{tm1a/tm1a}$ mice (related to Fig. 2). (A-D) Transverse sections through the cochlea of control and *Gas2*$^{tm1a/tm1a}$ mice at P14 (A, E) and P25 (B, F) stained for F-actin (green), dTyr α-tubulin (red) and DAPI (blue) to reveal inner and outer pillar cell morphology. **(E-H)** Whole mount cochlear preparations from control and *Gas2*$^{tm1a/tm1a}$ mice at P14 (E, G) and P25 (F, H) immunostained for MYO7A (red) to reveal inner and outer hair cell rows. A missing hair cell is indicated (asterisk). **(I-J)** The lengths of IPCs and OPCs were not significantly different between control and *Gas2*$^{tm1a/tm1a}$ mice (presented as mean ±SEM, ns=not significant, Student's t-test, n=3). Scale bar = 20μm. **(K-L)** The distance between OHC rows (dashed white line in E) and the distance between inner and outer hair cell rows (dashed blue line in E) was not significantly different between control and *Gas2*$^{tm1a/tm1a}$ mice (presented as mean ±SEM, ns=not significant, Student's t-test, n=3). (**M-S'**) Transverse sections through the stria vascularis of control (M-S) and *Gas2*$^{tm1a/tm1a}$ (M'-S') mice at P25 stained with layer specific markers as schematized in **(T)**. Despite the loss of GAS2 in the marginal cell layer, no other markers were differentially expressed between control and *Gas2*$^{tm1a/tm1a}$ mice. Scale bar = 25μm. **(U)** Endocochlear potential measurements between control (n=5) and *Gas2*$^{tm1a/tm1a}$ (n=4) mice at P56 were not significantly different (presented as mean ±SEM, p=0.8556, two-tailed t-test).

Figure S5

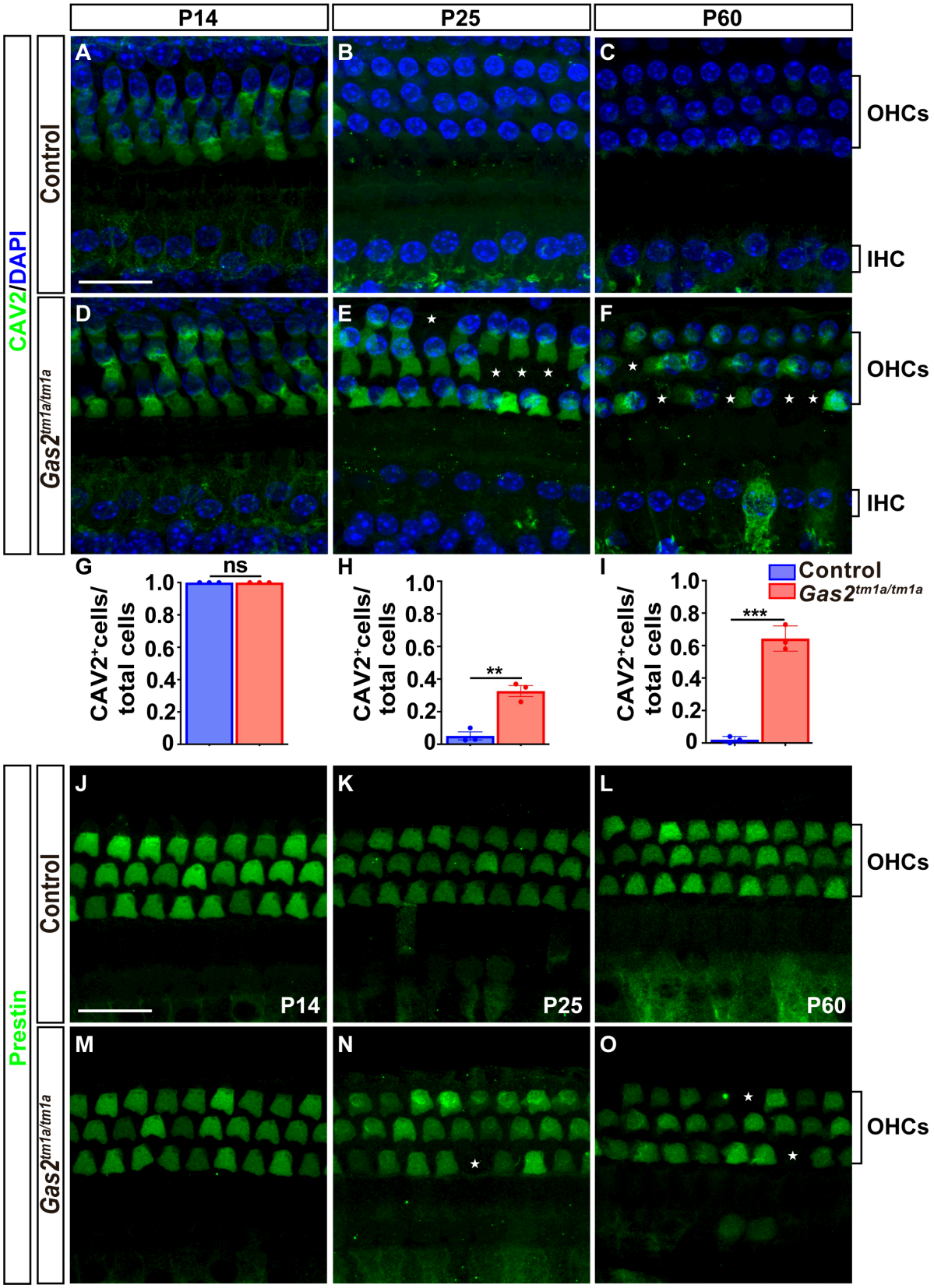
P14
P25
P60
CAV2/DAPI
Control
Gas2tm1a/tm1a
A
B
C
D
E
F
OHCs
IHC
OHCs
IHC
G
H
I
ns
**
***
CAV2+cells/ total cells
1.0
0.8
0.6
0.4
0.2
0
Control
Gas2tm1a/tm1a
Prestin
Control
Gas2tm1a/tm1a
J
K
L
M
N
O
P14
P25
P60
OHCs
OHCs

Figure S5. OHCs display increased mechanical stress and maintain Prestin expression in *Gas2*$^{tm1a/tm1a}$ mice (related to Fig. 5). (A-F) Whole mount cochlear preparations from control and *Gas2*$^{tm1a/tm1a}$ mice at P14 (A, D), P25 (B, E) and P60 (C, F) immunostained for Caveolin-2 (CAV2). Missing hair cells are indicated (asterisk). **(G-I)** Quantification of the ratio of CAV2^{+} cells to the total number of hair cells at P14, P25, P60 presented as mean ±SEM (ns=not significant, **p<0.01, ***p<0.001, two sided t-test, n=3). Whole mount cochlear preparations from control and *Gas2*$^{tm1a/tm1a}$ mice at P14 (J, M), P25 (K, N) and P60 (L, O) showing no difference in the expression of Prestin. Scale bar = 20µm.

Figure S6

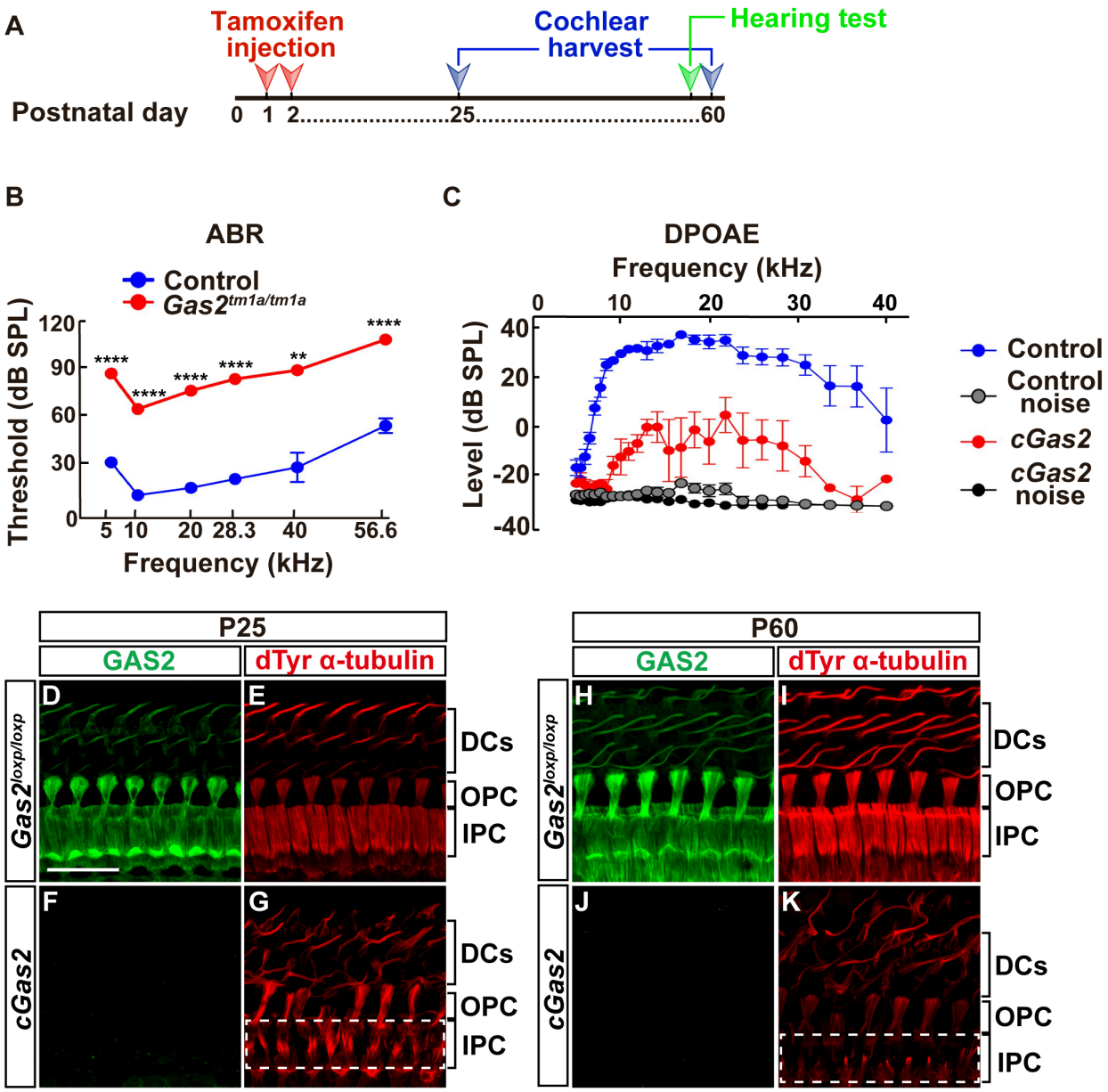
A
Tamoxifen injection
Cochlear harvest
Hearing test
Postnatal day
0 1 2.....25.....60
B
ABR
Control
Gas2tm1a/tm1a
Threshold (dB SPL)
120
90
60
30
0
****
****
****
****
**
****
5 10 20 28.3 40 56.6
Frequency (kHz)
C
DPOAE
Frequency (kHz)
0 10 20 30 40
Level (dB SPL)
40
20
0
-20
-40
Control
Control noise
cGas2
cGas2 noise
P25
P60
GAS2
dTyr α-tubulin
Gas2loxp/loxp
cGas2
D
E
F
G
H
I
J
K
DCs
OPC
IPC

Figure S6. GAS2 is required postnatally in supporting cells for microtubule stability and hearing (related to Fig. 5). (A) Timeline for the generation and analysis of *cGas2* knockout mice. **(B)** ABR thresholds from control (n=3) and c*Gas2* (n=3) mice at P60 (**p<0.01, ****p<0.0001, multiple T-test with Holm-Sidak method). **(C)** Reduced DPOAE response in *cGas2* mice at P60 (p<0.05, multiple T-test with Holm-Sidak method, n=3). **(D-K)** Whole mount cochlear preparations from control and c*Gas2* mice at P25 (D-G) and P60 (H-K) immunostained for GAS2 and detyrosinated α-tubulin. A progressive loss of pillar cell microtubules is observed with age, comparable to that described in $Gas2^{tm1a/tm1a}$ mice (Fig. 2G,H,L,M).

Figure S7

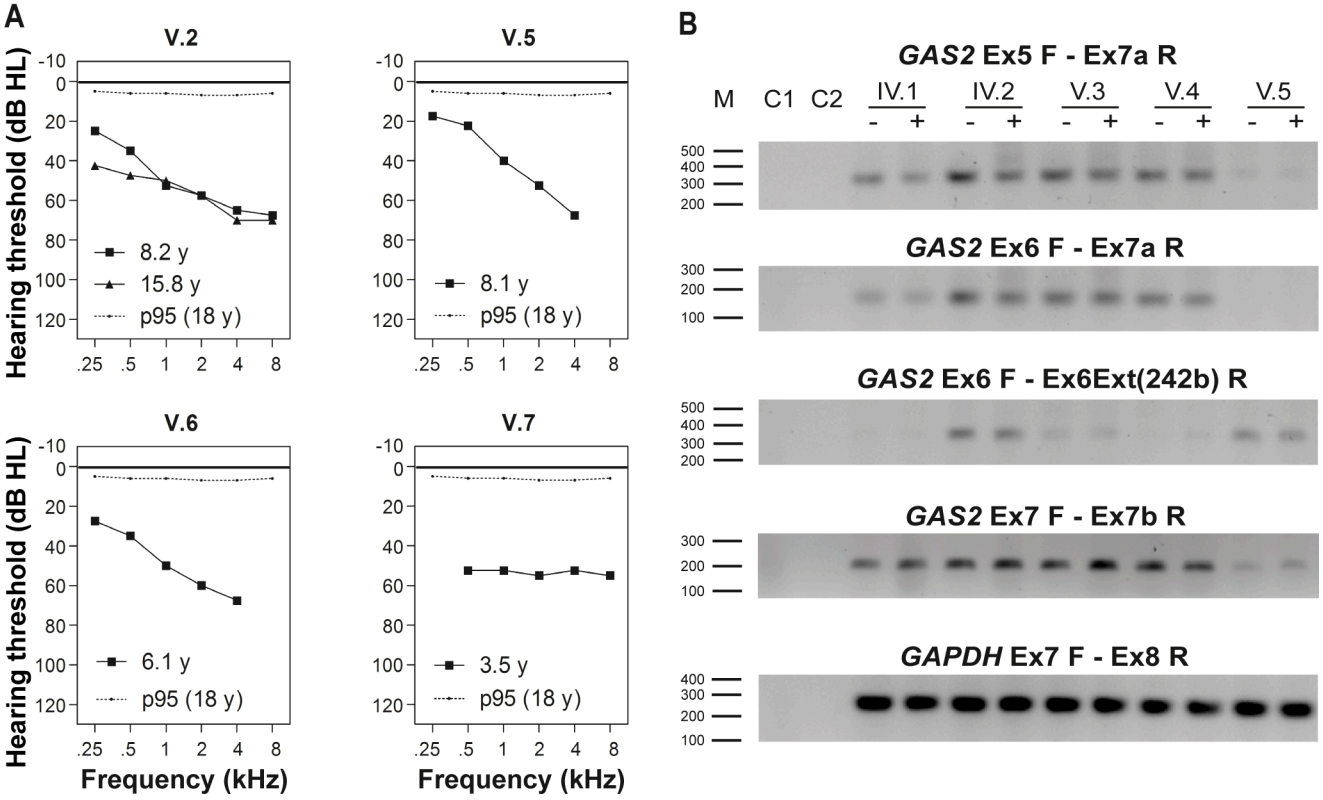

Figure S7. Hearing loss and splicing alterations in subjects with the c.723+1G>A *GAS2* variant (related to Fig. 7). (A) Average air conduction thresholds of subjects V2 and V.5-7 with the c.723+1G>A *GAS2* variant in the homozygous state. The dotted line represents the 95th percentile of age- and sex-specific hearing level at the age of 18 years. This is the lowest age for which the ISO 7029:2017 standard can be applied. Thresholds of subjects V5 and V6 are based on pure tone audiometry; thresholds of subject V7 are based on conditioned play audiometry. Age in years (y), decibel hearing level (dB HL), kilohertz (kHz). **(B)** RT-PCR using primer pairs in exon 6 and flanking exons and intronic sequences (referring to transcript NM_005256.3) was performed to detect aberrant splicing associated with the GAS2 c.723+1G>A variant. 100bp marker

(M), no reverse transcriptase in cDNA synthesis step (C1), PCR without cDNA input (C2), cells treated with cycloheximide (+), no cycloheximide treatment (-).

Figure S8.

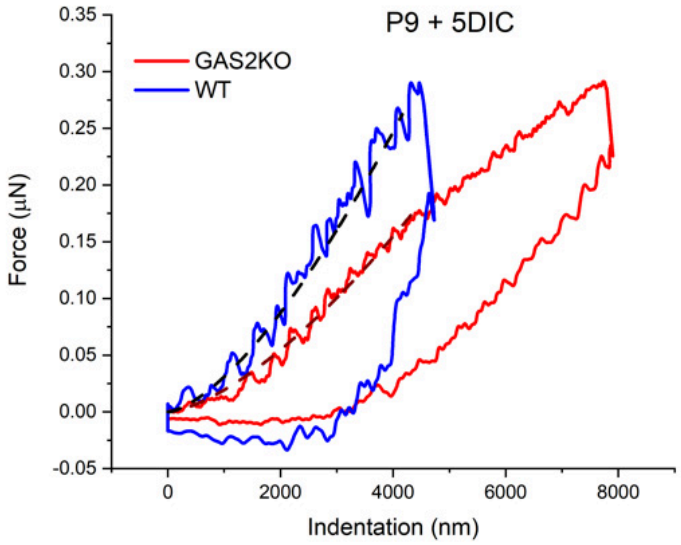

Fig. S8. Representative indentation curves from an atomic force microscopy experiment (related to STAR Methods: Atomic Force Microscopy and Fig. 4). Representative indentation curves from inner pillar cells taken from the Gas2 knockout (KO) and WT cochlea at P9 + 5DIC. The slope of the indentation (top portion of the curve) is fitted to the Hertz equation, dashed lines, for a spherical indenter to determine the stiffness of the cell.

Table S1. Primer sequences for analysis of human *GAS2* (related to Fig. 7 and Fig. S7)

Fragment	Primer name in Fig. 7 and Fig. S7		Oligonucleotides	RT-PCR size wildtype (bp)	RT-PCR size mutant (bp)
Primers for amplification and sequence analysis of genomic DNA					
GAS2 exon 6	NA	Forward:	GATGTCTTTGGGGCCTGAAC	-	-
	NA	Reverse:	GATCTCCATGGTGTCTTTGGTG		
Primers for RT-PCR and sequence analysis of *GAS2* transcript					
GAS2 exon 5 - exon 7	Ex5 F	Forward:	TTCTGCCCCTTCTCCTTCAC	294	-
	Ex7a R	Reverse:	ACACGGGAGATCTGCAGC		
GAS2 exon 6 - exon 7	Ex6 F	Forward:	GTTCTGTGTGGAGCGGCTC	171	-
	Ex7a R	Reverse:	ACACGGGAGATCTGCAGC		
GAS2 exon 5 - exon 6 extension c.723+242	Ex5 F	Forward:	TTCTGCCCCTTCTCCTTCAC	-	450
	Ex6Ext(242b) R	Reverse:	TCACAAGTGGAAAGCCTTCTTC		
GAS2 exon 6 - exon 6 extension c.723+242	Ex 6 F	Forward:	GTTCTGTGTGGAGCGGCTC	-	327
	Ex6Ext(242b) R	Reverse:	TCACAAGTGGAAAGCCTTCTTC		
GAS2 exon 6 extension c.723+241 - exon 7	Ex6Ext(241b) F	Forward:	TGAAGAAGGCTTTCCACTTGTG	-	-
	Ex7a R	Reverse:	ACACGGGAGATCTGCAGC		
GAS2 exon 6 extension c.724-224 - exon 7	Exon6Ext(-224b) F	Forward:	ACAGCAAAGCTGGGTTCTGG	-	331
	Ex7a R	Reverse:	ACACGGGAGATCTGCAGC		
GAS2 exon 7 – exon 7	Ex7 F	Forward:	TGCTGCACAACAAACATGTC	216	-
	Ex7b R	Reverse:	GGCAGAGACCACCAAGTAG		
GAPDH exon 7 - 8 (NM_002046.5)	NA	Forward:	CTGCACCACCAACTGCTTAG	219	-
	NA	Reverse:	AGCTCAGGGATGACCTTGC		

Primers for qPCR of *GAS2* cDNA					
GAS2 exon 3 - 4	NA	Forward:	ATCCTGGTGCCGAGATTTAGG	-	-
	NA	Reverse:	CACACTTCTCTGGGTTGCTTG		
GAS2 exon 6 - exon 6Ext	NA	Forward:	GTGGGAGAAAAGATCCTCTTC	-	-
	NA	Reverse:	GAAGTTCCTGCTCTTATCTGTC		
GUSB exon 2 - 3 (NM_000181.4)	NA	Forward:	AGAGTGGTGCTGAGGATTGG	-	-
	NA	Reverse:	CCCTCATGCTCTAGCGTGTC		

GAS2 reference sequence: NM_005256.3. (RT-)PCR was performed with annealing temperatures decreasing from 62.5 to 57.5 °C for 30 cycles. qPCR was performed with an anealing temperature of 60.0 °C.

Table S2. Individual results of newborn hearing screening, otoscopic examination, otoacoustic emissions and audiometry (related to Fig. 7).

Family	Subject	Newborn screening	Age of HL first detected (y)	Otoscopic examination	Otoacousic emissions				PTA		SRT		Maximum SRS (%)	
					TEOA	DPOAE	I/O	Age at hearing test (y)	R	L	R	L	R	L
W19-1278	IV.2	NA	NA	NT	present	present	present	38	8	5	NT	NT	NT	NT
	V.2	NT	1	N	absent	absent	absent	15	58	60	50	42	94	97
	V.3	NA	NA	NT	present	present	present	14	5	5	NT	NT	NT	NT
	V.4	NA	NA	NT	present	present	present	12	2	0	NT	NT	NT	NT
	V.5	P	1	N	absent	absent	absent	7	58	58	47	NT	93	98
	V.6	F	0	N	absent	absent	absent	5	60	62	45	50	95	95
	V.7	F	0	N	present	absent	absent	3	52	55	NT	NT	NT	NT

Age of onset (AoO), age of onset in years as reported by the subjects. Age at hearing test, the age at which the audiometric data of column 9 to 14 were obtained, in general the last audiogram. If no speech audiometry was performed during the latest pure tone audiometry, the latest audiogram in which both were measured, was selected. TEOAE, transient evoked otoacousic emissions; DPOAE, distortion product otoacoustic emissions; I/O, DPOAE input/output functions; y, years; PTA, pure tone average, mean of 0.5, 1 and 2 kHz air conduction thresholds; SRT, speech reception threshold; SRS, speech recognition score in %; R, right; L, left; NA, not applicable; NT, not tested; N, normal; P, pass; F, fail.

Table S3. Primer sets for genotyping mouse *Gas2* alleles (relates to Fig. S2)

Fragment	Primer name in Supplementary Figure 2		Oligonucleotides	PCR size wildtype (bp)	PCR size mutant (bp)
Primers for amplification and sequence analysis of genomic DNA					
5' arm	NA	Forward:	GAAGTTCCTATTCCGAAGTTCCT	7076	-
	NA	Reverse:	CTGCCTGTGTTGAATGTTCTCAT		
3' arm	NA	Forward:	CCTGCTTGCCGAATATCATGG	6493	-
	NA	Reverse:	ACTTCTGTGGAGTCTCAGCTC		
Tm1a/Loxp	NA	Forward:	TTGGATCATATGGAGAGAGCCAT	223	257
	NA	Reverse:	GGGCATATCACAGGCCCATA		
Tm1c	NA	Forward:	GGCGCATAACGATACCACGA	-	208
	NA	Reverse:	TATAAGCCGCCTACTGCGAC		
Gas2-LacZ	NA	Forward:	AGAACAAGCAACATGCGCTC	-	611
	NA	Reverse:	GACCTTGGGACCACCTCATC		
Gas2-Neo	NA	Forward:	CTCCCCCTGAACCTGAAACATA	-	360
	NA	Reverse:	TTATATAAGCCGCCTACTGCGA		
cGas2-Cre	NA	Forward:	GCGGCATGGTGCAAGTTGAAT	-	232
	NA	Reverse:	CGTTCACCGGCATCAACGTTT		